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Application of Fluorescence in Situ Hybridization to Detect N-myc (MYCN) Gene Amplification on Paraffin-Embedded Tissue Sections of Neuroblastomas

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Fluorescence in situ hybridization (FISH) was applied to neuroblastoma for detection of N-myc (MYCN) oncogene amplification, and the results were compared with Southern blot analysis (Southern). In nine neuroblastomas (formalin-fixed paraffin-embedded tissues were available in seven cases including two cases with touch preparations, and two cell lines), all five cases with N-myc amplification detected by Southern had cells with multiple N-myc signals by FISH, and three cases showed no N-myc amplification either by Southern or FISH procedure. One case, not examined by Southern, showed amplified signals of N-myc by FISH. These data indicate that FISH results for N-myc amplification have close correlation with Southern blot analysis. The chromosome 2-specific repetitive DNA probe was also applied for the analysis of ploidy by FISH. Six cases with N-myc amplification by Southern and/or FISH had diploid tumors and two cases without amplified N-myc showed aneuploidy. The remaining one case consisted of heterogeneous elements showing diploidy in undifferentiated tissue and both aneuploidy (ganglionic cells) and diploidy (Schwann cells) in differentiated area. We conclude that FISH is a practical, useful and reliable method over Southern especially for analysis of N-myc amplification in neuroblastoma, and simultaneous cohybridization with a specific chromosome probe is of great value in predicting the prognosis of patients. Med. Pediatr. Oncol. 29:135–138, 1997.
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Key words: neuroblastoma; fluorescence in situ hybridization; N-myc; ploidy

INTRODUCTION

Besides clinical stage, patients' age at diagnosis and histopathology, N-myc oncogene amplification is recognized as an independent reliable and unfavorable prognostic factor for identifying children with rapid progression of the disease and with the aggressiveness of the tumor [1,2]. In order to detect N-myc (MYCN) amplification by Southern blot analysis, it has been necessary to extract DNA from a sufficient amount of fresh tissue sample. Southern procedure requires somewhat complicated techniques and takes several days. The recent development of the fluorescence in situ hybridization (FISH) technique has introduced the rapid and sensitive detection of single copy DNA sequences in metaphase and interphase nuclei. In this study, we use FISH to detect N-myc gene amplification in neuroblastoma tissue including formalin-fixed paraffin-embedded tissues, cell lines and touch preparation specimens. The results by FISH were compared with those by Southern blot analysis. Also, the number of copies of chromosome 2 was examined since N-myc gene was located at chromosome band 2p23–24.

MATERIALS AND METHODS

Sample Preparation

Nine neuroblastomas were investigated in this study: Seven cases of surgically removed specimen including two cases with touch preparations, and two cell lines (NB-1 and NB-4; those were established in our laboratories). Three to five micron specimens were cut from formalin-fixed paraffin-embedded tissue and attached to glass slides coated with silan. These sections were deparaffinized in xylene and rehydrated through an ethanol series for 5 minutes. The slides were air-dried and incubated with 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7) at 72°C for 30 minutes. Next, digestion with 0.5% trypsin (Sigma, St. Louis, MO) in 0.01 M phosphate-buffered saline (PBS) was performed at 37°C for 30 minutes. This reaction was stopped by rinsing with 0.01 M PBS. Finally they were dipped in 50% formamide/2 × SSC.

Both touch preparations and the slides attached with cell suspension from cell lines were also air-dried and fixed in methanol/acetic acid (3:1) for 15 minutes. Then the slides were dried and incubated for 1 hour at 65°C

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TABLE I. Clinical Findings, N-myc Copy Number and FISH Results for Surgically Resected Neuroblastoma

Case	Age	Stage	Outcome	Histology[a]	*N*-myc[b] (copy number)	FISH	
						N-myc	Ploidy
1	1 y	IV	AWD[d]	Unfavorable	120	+	Diploid
2	5 m	IVS	Alive	Unfavorable	30	+	Diploid
3	3 y	IV	AWD	Unfavorable	ND[e]	+	Diploid
4	1 m	IV	Dead	Unfavorable	20	+	Diploid
5	3 y	IV	Dead	Unfavorable	1	−	Diploid + Aneuploid[c]
6	6 m	I	Alive	Favorable	1	−	Aneuploid
7	6 m	I	Alive	Favorable	1	−	Aneuploid

[a]Histology evaluated according to Shimada et al. [4].
[b]Number of N-myc copies by Southern blot analysis.
[c]Diploid in undifferentiated cells and Schwann cells and aneuploid in ganglionic cells.
[d]AWD, alive with disease.
[e]ND, not done.

and dehydrated with ethanol series and dried. Finally the slides were dipped in 50% formamide/2 × SSC.

In Situ Hybridization

We used a mixture of digoxigenin-labeled N-myc DNA probe/biotin-labeled chromosome 2-specific probe D2Z (20:1, Oncor, Gaithersburg, MD). This probe mixture was denatured in a water bath at 74°C for 5 minutes and immediately stored in ice. Pretreated slides were also denatured in 70% formamide/2 × SSC at 74°C for 10 minutes. Slides were then dehydrated in an ethanol series and air-dried. For each specimen, 10–15 microliters of the hybridization mixture were added and covered with a coverslip. Hybridization incubation was run at 37°C for overnight. After hybridization, the slides were washed at 72°C for 10 minutes in 2 × SSC and washed again at room temperature for 5 minutes in 2 × SSC.

Detection of Hybridized DNA Probe

Thirty microliter of rhodamine-labeled anti-digoxigenin (Oncor) was added and incubated at 37°C for 30 minutes. Then slides were washed in 2 × SSC three times. Next they were incubated at 37°C for 30 minutes in fluorescein-labeled avidin (Oncor) and washed in 2 × SSC three times. Counterstaining was accomplished with DAPI in anti-fade (100 mg p-phenylenediamine dihydrochloride in 10 ml PBS). Detected signals were observed by using Olympus fluorescent microscope without informed knowledge of the results of Southern blot analysis for N-myc gene amplification.

Southern Blot Analysis

DNA was isolated from two tumor cell lines (NB-1 and NB-4) by conventional techniques. After restriction digest of 5 micrograms of DNA per sample with EcoRI, the DNA was electrophoresed, transferred to a nylon membrane and hybridized to an insert probe isolated from pNB-1 that was labeled by the random primer technique to a specific activity of approximately 10^7 cpm/microgram. The filters were washed and exposed to X-ray film with an intensifying screen at room temperature overnight. Results of N-myc amplification on other cases were obtained from different laboratories and institutions.

TABLE II. N-myc and FISH Results for Two Neuroblastoma Cell Lines

Cell lines	N-myc (copy number)	FISH
NB-1	20	+
NB-4	30	+

RESULTS

Clinical findings and histologic classification according to Shimada [3] of seven patients are summarized in Table I. There were two cases of stage I, four stage IV and one IVS. Histologically, five cases were classified into an unfavorable and two were favorable histology group. Among five unfavorable histology groups, three cases (cases 1, 2, 4) were ''stroma-poor, undifferentiated, high mitosis-karyorrhexis index.'' One (case 3) showed ''stroma-poor, undifferentiated, low mitosis-karyorrhexis index,'' and was classified into an unfavorable group because of the patient's age at diagnosis [3]. Case 5 showed ''stroma-rich, nodular type'' neuroblastoma, which were consistent with so-called composite type of ganglioneuroblastoma (ganglioneuromatous tumor with grossly visible foci of undifferentiated neuroblastoma). Two tumors (cases 6 and 7) had a favorable histology of ''stroma-poor, undifferentiated, low mitosis-karyorrhexis index.''

Tables I and II compare the results of FISH analysis of nine neuroblastoma cases with findings by Southern blot

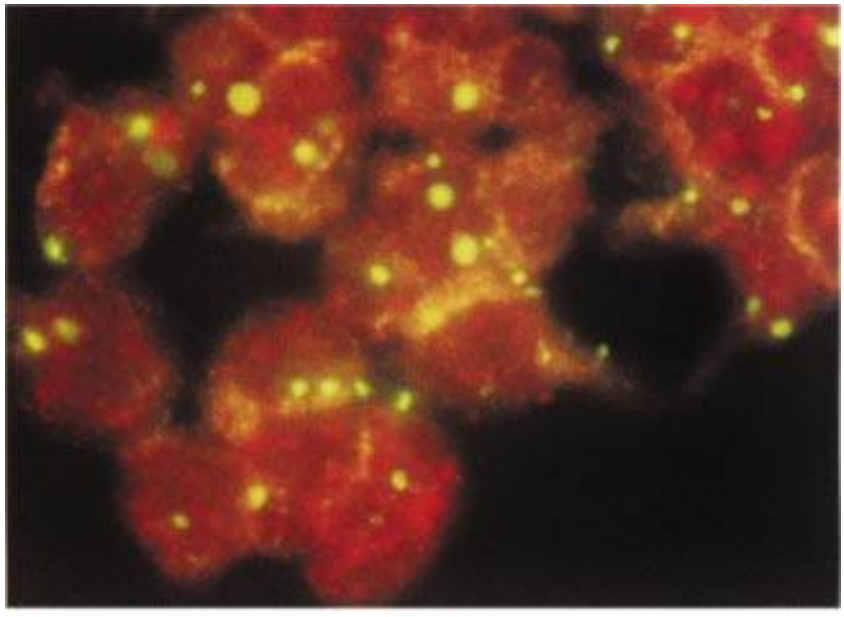

Fig. 1. Dual color FISH shows multiple N-myc signals (red dots) in most tumor cells, and also demonstrates one or two copies of chromosome 2 (yellow-green dots) in tumor cells (case 1).

analysis. Tumor cells prepared from three cases (cases 1, 2 and 4) of surgically removed specimen clearly showed multiple N-myc signals by FISH in each tumor nuclei (Fig. 1). Two neuroblastoma cell lines (NB-1 and NB-4) also have N-myc amplification. Intercellular heterogeneity in the extent of N-myc amplification by FISH was frequently seen within the same tumor specimen; that is, the number of N-myc signals was from one to numerous. Both tissue sections and touch preparation were examined in two cases (cases 6 and 7); one or two N-myc signals were identified in individual cells in both cases.

The positivity of N-myc amplification by FISH (+, the mean number of N-myc signals were three or more per cell; −, less than three signals per cell) consistently agreed with the results by Southern analysis. That is, all five cases of neuroblastoma (cases 1, 2, 4 and cell lines NB-1 and NB-4) with amplified N-myc detected by Southern showed positive results by FISH. In contrast, two cases (cases 6 and 7) showed no N-myc amplification by either Southern or FISH. Case 3, which had not been examined N-myc amplification by Southern, had multiple N-myc signals by FISH.

Based on the number of chromosome 2 signals in each tumor cell, tumors were classified into diploid (one or two copies of chromosome 2 per cell) and aneuploid (three or more copies of chromosome 2 per cell). Six cases (cases 1–4) showed diploidy and two cases (cases 6 and 7) were aneuploid tumor. One case (case 5), which was a histologically heterogeneous tumor (composite type of ganglioneuroblastoma), was diploid in an undifferentiated area. In differentiated tissue of this case, ganglionic cells showed aneuploidy, and Schwann cells had diploidy. In this study, all N-myc-amplified cases detected by FISH were diploid tumors. In contrast, aneuploid tumor showed no N-myc amplification.

DISCUSSION

We investigated the utility of FISH for detecting N-myc gene amplification in children with neuroblastoma. In 1993, Shapiro et al. [4] reported the detection of N-myc gene amplification by FISH on 20 neuroblastoma cell lines and 12 tissue sections. They demonstrated that FISH results were as accurate as Southern blot analysis. More recently, Taylor et al. [5] and Ambros et al. [6] also documented the advantages of FISH techniques to assess neuroblastomas for N-myc using touch preparations, bone marrow imprints and/or formalin-fixed paraffin-embedded tissues. Our study definitely supports their data and furthermore addresses the advantage of FISH technique over Southern blot analysis in terms of faster speed and simplicity, and the ability to demonstrate intercellular heterogeneity in N-myc amplification within the same tumor tissue.

It is well known that neuroblastoma sometimes contains foci or a mixture of differentiated area(s) in the background of undifferentiated tissue. In this study, the presence of intercellular heterogeneity of N-myc copy number within a tumor was an interesting observation. We speculate that neuroblastoma cells without N-myc amplification may have a potential of differentiation based on the fact that most differentiated neuroblastoma or ganglioneuroblastoma have no N-myc amplification.

Composite type of ganglioneuroblastoma or ‘‘nodular

type of stroma-rich neuroblastoma'' according to Shimada classification [3] was recognized as a peculiar tumor having grossly discrete mass(es) of undifferentiated element in ganglioneuromatous tissue. Shimada et al. [3] and Aoyama et al. [7] demonstrated the characteristic behavior of this tumor biologically and clinically. They speculated that the undifferentiated area might be interpreted as the malignant growth of aggressive clone(s) evolving late in the course of maturation. In 1986, Gansler et al. [8] reported flow cytometric DNA analysis of neuroblastoma. In their study, two cases of this heterogeneous tumor were examined and were proven to be an unfavorable pattern of ''euploid (diploid).'' Examined tissue in their study might be taken from only undifferentiated areas because unfavorable neuroblastoma was usually associated with diploid DNA pattern and most ganglioneuromas have aneuploidy [9–11]. In this study, we analyzed one case (case 5) of this unique tumor, showing diploidy in undifferentiated cells, aneuploidy in ganglionic cells and diploidy in Schwann cells, suggesting that composite ganglioneuroblastoma may be composed of two or more different clones. In such a heterogeneous tumor, FISH technique, using thin-cut sections on glass slides from routinely processed paraffin-embedded tissue, is of great value rather than using single cell suspensions or touch preparation.

Although only seven cases were examined in our study, clinical, histological and FISH results were closely correlated with each other. Based on the combination of these findings, seven cases in this study were biologically classified into two groups: five cases (cases 1–5) of the poor prognosis group and two (cases 6 and 7) of the good prognosis group. Brodeur and Nakagawara [12] proposed three subsets of neuroblastoma based on the results of clinical behavior and genetic abnormalities. According to their subclassification, five cases of poor prognosis group and two good prognosis group in our study can be classified as ''group 3'' and ''group 1,'' respectively.

Conclusively, with the use of FISH, dual independent prognostic factors of N-myc amplification and DNA ploidy can be examined at the same time. FISH is of value in predicting the prognosis of patients with neuroblastoma and its simplicity and faster speed is beneficial in the introduction of suitable treatment of neuroblastoma.

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REFERENCES

1. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM: Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224:1121, 1984.
2. Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D: Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 313:1111, 1985.
3. Shimada H, Chatten J, Newton WA, Sachs N, Hamoudi AB, Chiba T, Marsden HB, Misugi K: Histopathologic prognostic factors in neuroblastic tumors: Definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. JNCI 73:405, 1984.
4. Shapiro DN, Valentine MB, Rowe ST, Sinclair AE, Sublett JE, Roberts WM, Look AT: Detection of N-myc gene amplification by fluorescence in situ hybridization. Diagnostic utility for neuroblastoma. Am J Pathol 142:1339, 1993.
5. Taylor CPF, McGuckin AG, Bown NP, Reid MM, Malcolm AJ, Pearson ADJ, Sheer D: Rapid detection of prognostic genetic factors in neuroblastoma using fluorescence in situ hybridization on tumour imprints and bone marrow smears. Br J Cancer 69:445, 1994.
6. Ambros PF, Ambros IM, Strehl S, Bauer S, Luegmayr A, Kovar H, Ladenstein R, Find FM, Horcher E, Printz G, Mutz I, Schilling F, Urban C, Gadner H: Regression and progression in neuroblastoma. Does genetic predict tumour behavior? Eur J Cancer 31A: 510, 1995.
7. Aoyama C, Qualman SJ, Regan M, Shimada H: Histopathologic features of composite ganglioneuroblastoma. Immunohistochemical distinction of the stromal component is related to prognosis. Cancer 65:255, 1990.
8. Gansler T, Chatten J, Varello M, Bunin GR, Atkinson B: Flow cytometric DNA analysis of neuroblastoma. Correlation with histology and clinical outcome. Cancer 58:2453, 1986.
9. Taylor SR, Blatt J, Costantino JP, Roederer M, Murphy RF: Flow cytometric DNA analysis of neuroblastoma and ganglioneuroma. A 10-year retrospective study. Cancer 62:749, 1988.
10. Look AT, Hayes FA, Shuster JJ, Douglass EC, Castleberry RD, Bowman LC, Smith EI, Brodeur GM: Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: A Pediatric Oncology Group Study. J Clin Oncol 9:581, 1991.
11. Naito M, Iwafuchi M, Ohsawa Y, Uchiyama M, Hirota M, Matsuda Y, Iinuma Y: Flow cytometric DNA analysis of neuroblastoma: Prognostic significance of DNA ploidy in unfavorable group. J Pediatr Surg 26:834, 1991.
12. Brodeur GM, Nakagawara A: Molecular basis of clinical heterogeneity in neuroblastoma. Am J Pediatr Hem/Onc 14:111, 1992.